COLLABORATIVE RESEARCH PROGRAM ON SEAFOOD TOXINS

FINAL REPORT

SAMUEL W. PAGE

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FOREWORD

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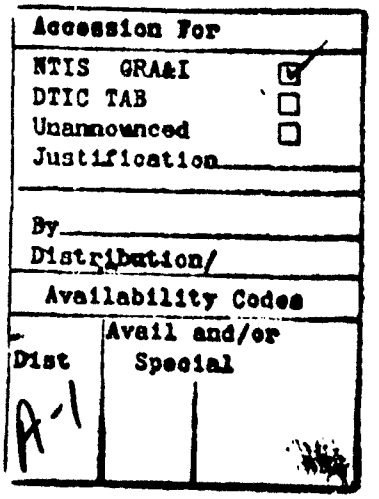

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INTRODUCTION

This Interagency Agreement for Collaborative Research on Seafood Toxins has contributed substantially to advances that are important to both the food safety mission of the U.S. Food and Drug Administration and to the medical defense against biological warfare mission of the Department of Defense. Significant progress has been made in all areas of the Scope of Work. These are summarized in the Workscope Accomplishments. Collaborative studies with DOD have continued following the termination of this formal agreement. We gratefully acknowledge the support and encouragement of Colonel David L. Bunner, M.D., and Robert W. Wannemacher, Jr., Ph.D., of the U.S. Army Medical Research Institute of Infectious Diseases.

The following individuals participated in the described studies (NPIB = FDA Natural Products and Instrumentation Branch)

Samuel W. Page, Ph.D. (Project Director) (NPIB)
Sherwood Hall, Ph.D. (Principle Investigator) (NPIB)

Robert Dickey, Ph.D. (FDA Fisheries Research Branch, Dauphin Island, A
Mohamed N. Gomaa (NPIB)
James Hungerford, Ph.D. (FDA Seafood Products Laboratory, Seattle, WA)
Ellen W. King (NPIB)
Roger Mann, Ph.D. (Virginia Institute of Marine Science)
Edward Moczydlowski, Ph.D. (Yale University School of Medicine)
Edward P. Ragelis, Ph.D. (NPIB)
Julia Rainer (Virginia Institute of Marine Science)
Gary R. Strichartz, Ph.D. (Harvard Medical School)
John J. Sullivan, Ph.D. (FDA Seafood Products Laboratory, Seattle, WA)
Mark Tamplin, Ph.D. (FDA Fisheries Research Branch, Dauphin Island, A
George C. Yang, Ph.D. (NPIB)

WORKSCOPE ACCOMPLISHMENTS

I. Determination of Purity of Paralytic Shellfish Poisons (PSP) Reference Standards

A. Nuclear Magnetic Resonance Spectroscopic Studies

Nuclear magnetic resonance (NMR)spectroscopic studies have been carried out on 12 of the PSP toxin standards and on domoic acid. These studies have included proton, carbon-13, and nitrogen-15 NMR spectral measurements. Extensive studies of the changes in the molecular structures of saxitoxin and neosaxitoxin with variations in pH were also carried out.

The NMR studies of the saxitoxins included efforts to quantify the toxins, both to determine epimer ratios of the 11-hydroxysulfate toxins and to determine the absolute concentrations of some of the synthetic saxitoxin derivatives produced for pharmacological characterization. Notwithstanding the intrinsic difficulties of NMR quantitation, these efforts were fairly successful, establishing concentrations or concentration ratios within better than 10% accuracy.

The preparation of a manuscript describing these studies is in progress. The description of the NMR spectroscopic techniques are given in Appendix A.

B. Optical Rotation Studies

Optical rotation was used as a measure of toxin purity in the classical studies of saxitoxin purification and has been neglected since, given the availability of more sophisticated techniques. This technique was evaluated during the present studies and found to be one of the better techniques for evaluating the absolute purity of saxitoxin. The optical rotations of pure saxitoxin preparations agreed well with the previously reported value, $[\alpha]^{25} = 130°$.

The limiting factor in such determinations turns out to be the accuracy with which the dry weight of the saxitoxin can be determined. The purification of saxitoxin now involves repeated chromatographic steps which provides a purified product, which is a glassy solid. The purity of this product must be independently determined. If the final saxitoxin preparation were a crystalline solid, rather than a amorphous solid, its purity and ease of manipulation would be significantly enhanced. There are two published accounts in which saxitoxin has been crystallized: as the ethyl hemiketal dihydrochloride and as the di-p-bromobenzenesulfonic acid salts. The latter approach has been evaluated and found to be less than ideal for the purposes of purification or characterization because of the small size of the resulting crystals and their relatively high solubility. Due to the high solubility, these crystals can only be obtained from highly concentrated solutions, so considerable difficulties were encountered in removing impurities present in the mother liquors. Current and future efforts will focus on optimizing these approaches and on the search for better acid salt.

C. X-Ray Crystallographic Structure Determinations

The crystal structures of saxitoxin, C2, and C4 had been previously determined and have provided important insights into the detailed structure of the toxins. However, the resolution obtained in the previous C2 X-ray crystallographic analysis was of poor quality. In addition, none of these previous studies defined the orientation of the 11-alpha-hydroxyl substituent. This information is of considerable importance to the understanding of the chemistry and pharmacology of the saxitoxins. After several efforts, crystals of saxitoxin C1 (an 11-alpha-hydroxysulfate) and C3 suitable for an X-ray crystallographic structure determination were obtained. In collaboration with the Naval Research Laboratory, the crystal structures for C1 and C3 were determined for the first time. In addition, a higher quality structure for C2 was obtained. These data sets, in conjunction with the NMR data, will be of great importance in our continuing efforts to model the active conformations of the saxitoxins and their interactions with the sodium channel binding site. Computer-generated crystal structures for C1 and C2 are given in Appendix B.

2. Investigations of the Modes of Actions of the Individual PSP Toxins

Collaborative studies of the molecular basis for the action of the PSP toxins were carried out with Dr. Gary R. Strichartz at Harvard Medical School and with Dr. E. Moczydlowski at the School of Medicine at Yale University.

The efforts to characterize the interaction between saxitoxin and its binding site and to develop a suitable non-exchangeable radiolabelled saxitoxin derivative have focused on the synthesis of saxitoxin derivatives and the study of their pharmacology.

Alpha-saxitoxinol was produced using literature methods and was found to have intrinsic activity using the bilayer technique. This lays to rest the uncertainty regarding previous experiments which employed binding or electrophysiological methods that could not distinguish between intrinsic toxicity at low level and contamination of a non-toxin derivative with traces of an active toxin.

Acetyl decarbamoylsaxitoxin was synthesized and found to be active using both binding and single channel techniques. This derivative is formally equivalent to the replacement of the terminal $-NH_2$ of the carbamate side chain with a $-CH_3$. This result suggests that, not withstanding the importance of the 21-sulfo group, hydrogen bonds to the terminal nitrogen are not essential for binding.

Decarbamoyl neosaxitoxin was synthesized and, quite to our surprise, revealed little detectable activity in either the binding or single channel assays. This is quite interesting, since it lies far outside the envelope of additive effects of the two functional modifications taken separately. These results suggest that there is an intramolecular interaction that is negatively synergistic with respect to binding.

3. Development/Evaluation of Rapid and Sensitive Analytical Methods for the Detection, Quantitation, and Confirmation of Seafood Toxins

An HPLC method for the PSP toxins was extensively evaluated, including a extramural contract with the State of Massachusetts, Theobold Smith Research Institute. In addition, a mass spectrometric procedure and a radioimmunoassay for PSP were also evaluated. The Hokama stick test for ciguatera toxin was also evaluated.

4. Initiate Studies on the Accumulation and Release of PSP Toxins by Administering Microencapsulated, Radiolabelled Toxins to Bivalves under Defined Conditions

This work was done in collaboration with an extramural contractor.

5. Provide PSP Reference Standards to DOA for Evaluation of Analytical Methods.

The following standards have been delivered to DOA:

saxitoxin:	829 mg
neosaxitoxin:	43 mg
B1:	3.7 mg
B2:	1 mg
C1:	18.5 mg
C2:	15.8 mg

6. Establish Culturing Facility for Dinoflagellates to Produce Ciguatoxin-related Compounds to Be Used for Structure Elucidation and for Development of Analytical Methodology.

A culturing facility for tropical dinoflagellates has been established by Dr. Robert Dickey at the FDA Division of Contaminants Chemistry, Fisheries Research Branch Facility, Dauphin Island, AL. The progress of these studies is given in Appendix C.

7. Organize a Workshop on Dinoflagellate Toxins with Emphasis on Analytical Methodology.

A workshop "Natural Toxins from Aquatic and Marine Environments" was held 27-30 August 1987 at the Marine Biological Laboratory at Woods Hole, Massachusetts. The proceedings of this conference were published by the American Chemical Society in March 1990. Six copies of this book were delivered to DOA on 26 April 1990.

8. Provide Consultation

Consultative services have been provided on request. In addition, field samples from several episodes of human toxicoses related to marine toxins were provided to DOA.

APPENDIX A

Characterization of PSP Toxins by Nuclear Magnetic Resonance Spectroscopy

EXPERIMENTAL

NMR spectra of STX and NEO were recorded in 5% D_2O/95% H_2O at various pH values on a Varian Associates VXR-400 spectrometer for the following nuclei: ^{1}H(400 MHz), ^{13}C(100 MHz), and ^{15}N(40.6 MHz).

Proton spectra, described by 1344 data points (real part), were obtained at 400 MHz using presaturation delay times of 10 sec. Pulse widths of 10 μs were employed, which correspond to tip angles of 22° with 5-mm sample tubes. A 10-dB attenuator was used to prevent receiver overload under these signal acquisition conditions. Spectral widths of 1400 Hz were employed, corresponding to acquisition times of *ca.* 0.5 sec. Chemical shifts are reported relative to the water signal at 4.6 ppm.

Proton-decoupled ^{13}C spectra, described by 32,768 data points (real part), were obtained with broad-band irradiation at 400 MHz. Pulse widths of 7 μs were employed, which correspond to tip angles of 35° with 5-mm sample tubes. Spectral widths of 20 kHz were used, corresponding to acquisition times of ca. 0.82 sec. Data were subjected to a 1-Hz line broadening, and chemical shifts are reported relative to the C-12 resonance at 99 ppm.

Proton-decoupled ^{15}N spectra, described by 32,768 data points (real part), were obtained with STX with broad-band irradiation at 400 MHz. Pulse widths of 10 μs were employed, which correspond to tip angles of 35° with 5-mm sample tubes. Spectral widths of 6 kHz were used, corresponding to

acquisition times of ca. 2.73 sec. STX data were subjected to a 0.4-Hz line broadening. Proton-decoupled ^{15}N spectra, described by 16,192 data points (real part), were obtained for NEO with gated (nOe-suppressed) broad-band irradiation at 400 MHz. Pulse widths of 24 μs were employed, which correspond to tip angles of 90° with 5-mm sample tubes. Spectral widths of 8 kHz were used, corresponding to acquisition times of ca. 1 sec. A pulse recycle time of 11 sec. was employed, and the NEO data were subjected to a 1-Hz line broadening. A 2.9 M $^{15}NH_4Cl$ solution was used as an external standard for both compounds, and chemical shifts are reported relative to the $^{15}NH_4^+$ resonance at 24.93 ppm.

STX/NEO

DISCUSSION

^{15}N NMR is well suited to address the question of deprotonation order, upon basification, of the guanidinium groups of STX and NEO because it provides a direct probe of the nitrogen atoms which are involved in these processes. Crucial to the interpretation of pH-^{15}N chemical shifts profile data are, of course, assignments of resonances of the nitrogen nuclei which comprise these two guanidinium groups, viz. nitrogens 1,3,15 and 7,9,16. These assignments were made at pH 3 and 7 by means of ^{13}C-^{15}N chemical shift correlation experiments. These assignments were then extended to other pH values by observing intensities and multiplicities of the nitrogen signals in question.

The carbon chemical shifts, on which these nitrogen shift assignments are based, are grouped into three categories for purposes of assignment and were identified by the following methods: (i) APT experiments and chemical shift considerations differentiated carbons 4,11, and 12; (ii) direct heteronuclear chemical shift correlation (HETCOR) experiments distinguished carbons 5,6,10, and 13, and (iii) indirect heteronuclear shift correlation (FLOCK) experiments identified carbons 2,8, and 20. To elaborate, the three quaternary-sp^3 carbons (4,11, and 12) were easily differentiated because C-11 appears as a quintet due to deuterium coupling upon exchange of the labile 11-protons while C-12 has a distinctive geminal-diol resonance near 100 ppm. The protonated carbons were, likewise, readily assigned by HETCOR experiments because their directly-attached protons (5,6,10, and 13) are themselves unambiguously identified by proton chemical shift correlation (COSY) experiments: protons 6,13A, and 13B comprise a relatively isolated 3-spin system since the dihedral angle between H-5 and H-6 is approximately 90°, and $^3J_{H_5H_6}$ is, therefore, essentially zero. H-5 appears as a slightly broadened singlet while protons 10A and 10B are observed as an isolated AB-system.

Assignment of the three quaternary-sp^2 carbons (2,8, and 20) was more difficult because their chemical shifts are very similar. However, FLOCK experiments revealed that C-2 is coupled to H-6 and H-10A. In addition, this carbon appears as a doublet of doublets in fully-coupled spectra. C-8 is

coupled to H-5 and, correspondingly, is observed to be a doublet under conditions of full coupling. C-20 is coupled to both 13-protons and appears as a triple in coupled carbon spectra. Like the ^{15}N spectra, assignments of

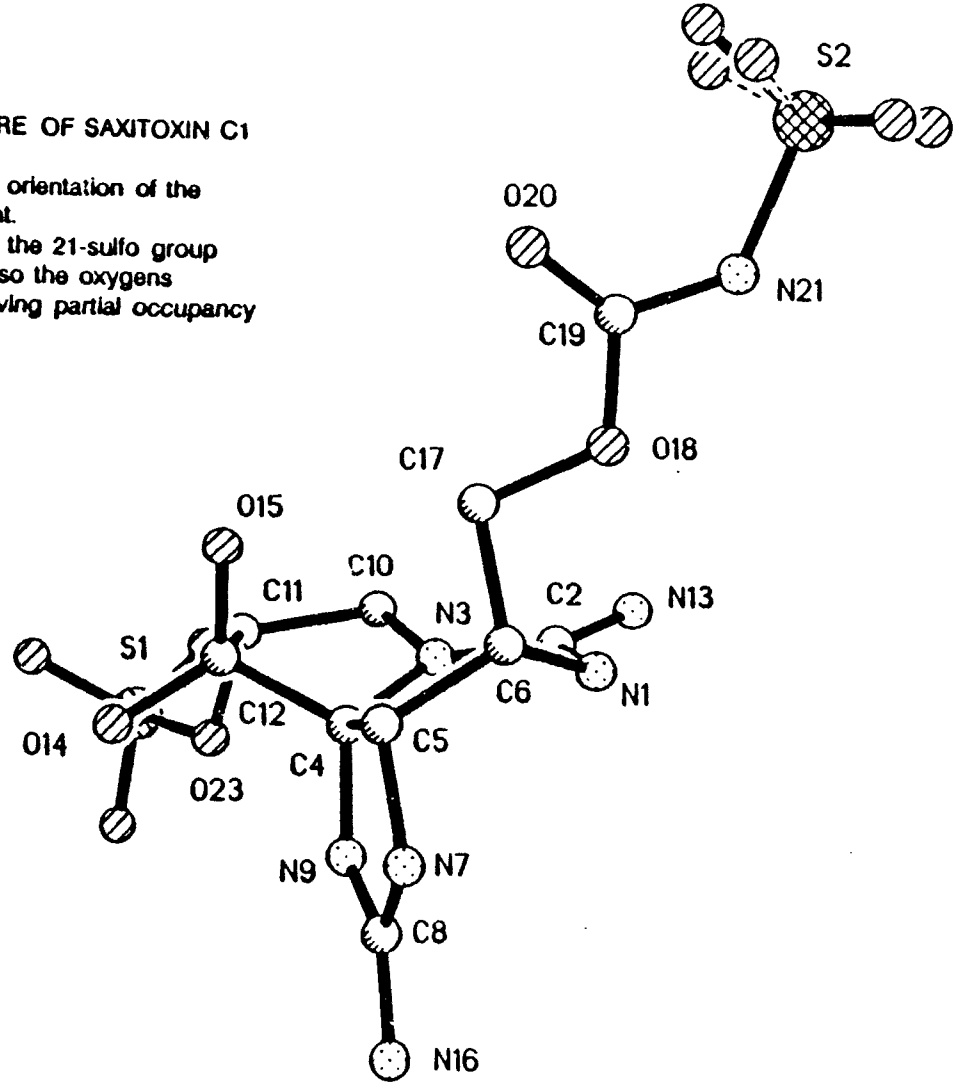
this last group of carbons were extended to other pH values by observing multiplicities of the carbon resonances in question in fully-coupled carbon spectra.

Upon establishment of the assignments of the seven ^{15}N signals over pH ranges which encompassed one, or both, pK_a values of STX and NEO, the relative deprotonation order could be determined for the two quanidinium groups of each toxin. As the pH of an aqueous solution of STX was raised from 4 to 11, resonances ascribable to nitrogens 7,9, and 16 were observed to undergo changes in chemical shift of ca. 4.5,50, and -11 ppm respectively, while those of the remaining four nitrogens exhibited less change individually (N-21) or as a group (1,3,15). Signals due to the dehydro, 12-keto form of STX, which predominates over the 12-diol form at higher pH, appeared for various nitrogens between pH 8.5-9.5. The above pH-chemical shift profile indicates that the C-8 quanidinium group of STX deprotonates at lower pH (PKa approx. 8.6) while the C-2 quanidinium group undergoes deprotonation at considerably higher pH.

The order of depronation for NEO is the reverse of that of STX: nitrogens 1,3, and 15 exhibited changes in chemical shift of ca. 14,-12, and -12 ppm, respectively, over the range 4-8.5. At the higher pH range 6-11, nitrogens 7,9, and 16 displayed chemical shift changes of ca. 5,43, and -11 ppm, respectively. The concerted movement of ^{15}N resonances of first the C-2 quanidinium group and then the C-8 group suggests pKa values for the two units of 7.2 and 9.25, respectively. As in STX, the keto-diol equilibrium of NEO shifts in favor of the keto form at high pH.

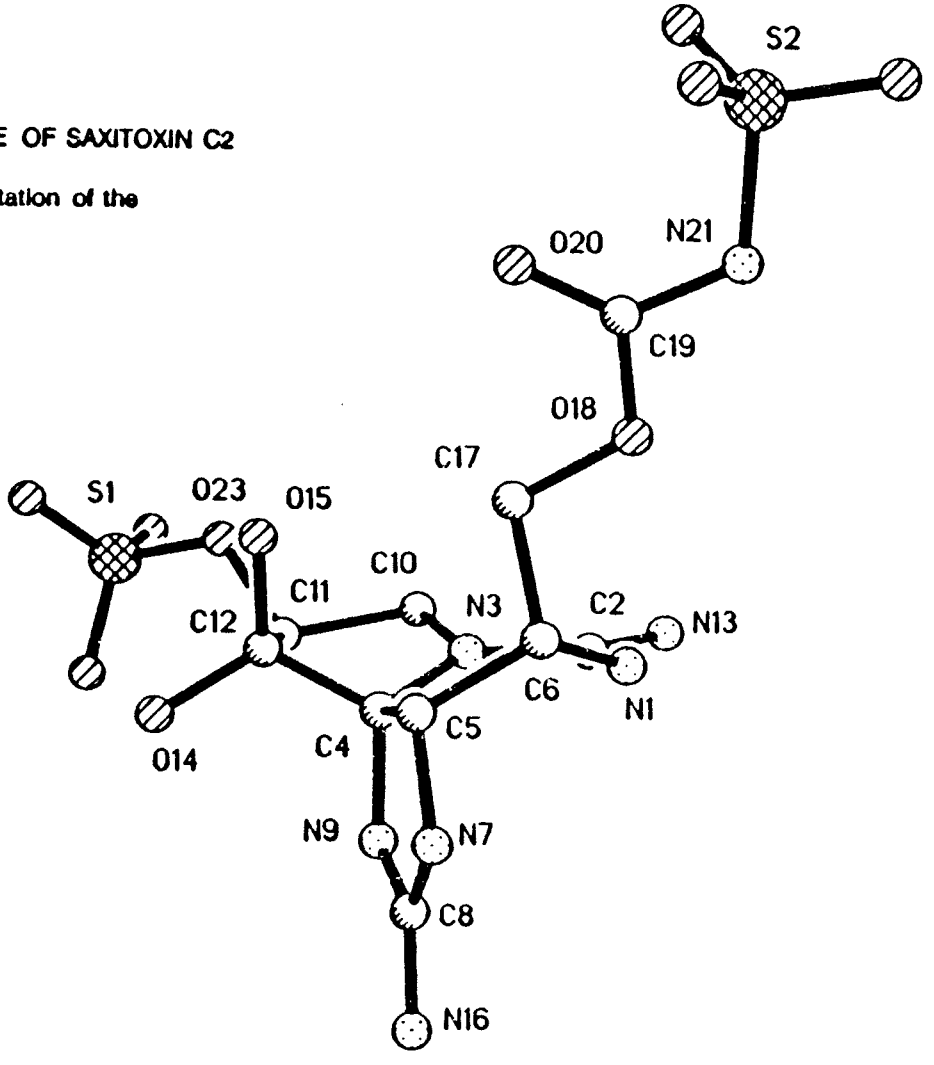
X-RAY CRYSTAL STRUCTURE OF SAXITOXIN C1

Note the alpha (downward) orientation of the 11-hydroxysulfate substituent.
The rotational orientation of the 21-sulfo group varies in the crystal lattice, so the oxygens are represented here as having partial occupancy of two sites.



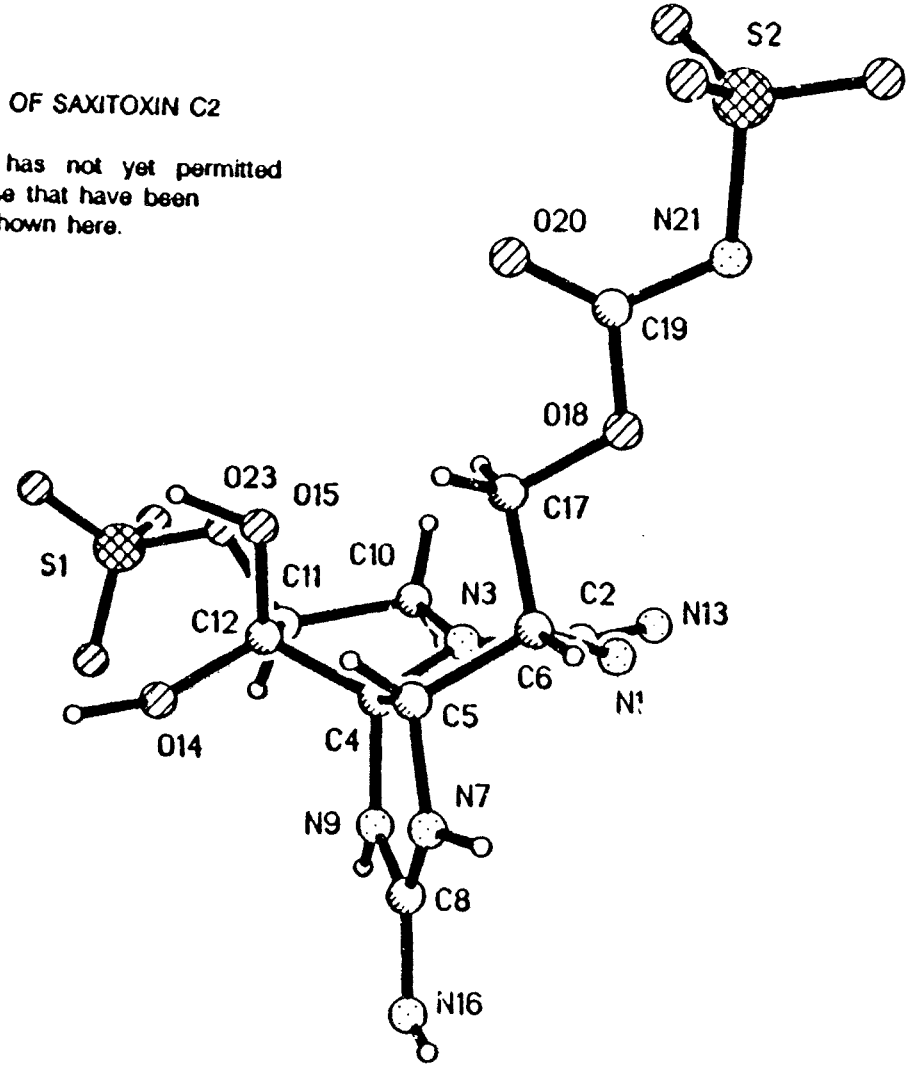
X-RAY CRYSTAL STRUCTURE OF SAXITOXIN C2

Note the beta (upward) orientation of the 11-hydroxysulfate substituent.



X-RAY CRYSTAL STRUCTURE OF SAXITOXIN C2

The quality of the crystals has not yet permitted resolution of all protons. Those that have been resolved in work to date are shown here.



APPENDIX C

COLLABORATIVE RESEARCH PROGRAM ON SEAFOOD TOXINS
PROGRESS REPORT ON CIGUATERA AND RELATED TOXINS

Finfish which enter the U.S. seafood supply from tropical and subtropical regions of the world are known to occasionally harbor natural toxins which cause a form of human poisoning referred to as ciguatera. The responsible toxins originate from unicellular marine algae of the division Pyrrhophyta (commonly known as the dinoflagellates) and more specifically from benthic and epiphytic species of this division. Dinoflagellates comprise a substantial proportion of the marine food base in warm-water oceans and are thus direct or indirect dietary components of finfish from these regions. The potential for human poisoning due to ciguatera-related toxins is a significant public health and socioeconomic threat. Therefore, it is essential that the responsible toxins are identified and that effective and practical detection methods are developed. The objective of this project is to determine the number and chemical identity of toxins associated with ciguatera fish poisoning and to develop methodology for the detection of these toxins in seafood.

The isolation of ciguatera-related toxins from finfish is the preferred approach to identification and methods development. However, because of the difficulty and inconsistency encountered in obtaining toxic fish specimens, the isolation of toxins from progenitors (dinoflagellates) is considered a viable alternative for identifying the toxins responsible for ciguatera. The dinoflagellate species Gambierdiscus toxicus Adachi

and Fukuyo, Protocentrum concavum Fukuyo, P. lima (Ehrenberg) Dodge, Ostreopsis siamensis Schmidt and O. lenticularis Fukuyo are the most strongly implicated sources of ciguatera-related toxins based upon their distributions, natural abundances and characteristics of toxicity. The foundation of this project is the dinoflagellate culture collection of Dr. Robert Dickey, which includes multiple strains (different time and geographic isolates) of each of the species listed above with the exception of O. siamensis. Other toxigenic species isolated from warm-water oceans also are included in this collection, but these species are thought to be of lesser significance in the etiology of ciguatera.

Toxins of Gambierdiscus toxicus

The species Gambierdiscus toxicus is reputed to produce ciguatoxin as described and structurally characterized from the moray eel (Lycodontis javanicus; Tachibana et al., 1981 & Murata et al., 1989). Murata et al. 1989, also describe ciguatoxin from G. toxicus cells collected from a field location as part of a detrital mixture. The latter finding has not been substantiated using cultured cells. Rather, the literature is replete with accounts of the absence of ciguatoxin-like compounds from cultured cells. The findings of this project also indicate a lack of ciguatoxin-like compounds from cultured G. toxicus. However, two lipid-soluble toxins have been isolated from cultured cells. The quantities of the two purified fractions were small, 0.40 mg and 0.15 mg, and complete structural elucidation was not possible. The toxins were completely soluble in DMSO and methanol; chloroform also appeared to be a good solvent, while acetone and acetonitrile were poor solvents. Proton NMR (200 MHz, 800 to 6,700 scans in deuteriochloroform) indicated that the

two molecules were very similar in structure. Neither appeared to resemble polyethers of the brevetoxins, okadaic acid or ciguatoxin class, but rather spectral interpretation of the limited data indicated that they might be fully substituted polycyclic or heteroaromatic compounds. Bromine or chlorine are presently thought to be likely candidates for some of these substituents. The UV spectra support in part the foregoing interpretation. Proton NMR provided limited information: both molecules have two methoxy groups (singlets at 3.6), 3-4 protons attached to carbon bearing oxygen (multiplets from 3.4-3.5), one methyl group (doublet at 1.1) coupled to at least one proton, and one proton which appears in a strained arrangement (multiplet at 0.9). The mass spectra of the molecules were not particularly informative and carbon NMR was unsuccessful. The accumulation G. toxicus biomass from culture in order to isolate sufficient purified toxins to complete this investigation is underway.

Toxins of Protocentrum concavum & P. lima

The discovery of the marine toxin okadaic acid (OA) from a Tahitian strain of P. lima was notable as the first toxin to be fully characterized from a dinoflagellate implicated as a source of ciguatera-related toxins (Murakami et al., 1982). The symptomatological and physical properties of OA resembled those of the then partially characterized ciguatoxin molecule from moray eel (Tachibana et al., 1981), however, the significance of OA in the etiology of ciguatera was generally thought to be limited.

During the last quarter of 1988, OA was isolated and identified from cultured P. concavum and shortly thereafter confirmed OA production in P. lima using analytical methodology to be described below. Positive structural confirmation of OA from P. concavum was provided by proton NMR, and EI, +/-FAB and +/-CI mass spectrometry of the underivatized and the trimethylsilyl and pentafluoro-benzyl derivatized toxin. The identification of OA from P. concavum was a first reporting for this species and also represents the first and only ciguatera-related toxin to be structurally characterized from the tropical Atlantic. Positive and negative ion FAB spectra of three other compounds isolated during the P. concavum study appear identical to the spectra of OA. However, hydroxyl ion negative CI spectra exhibited minor but distinct differences from those of the OA OH-/NCI spectrum. Although all four compounds provided an intense response at m/z 803 $[M-H]^+$, all but OA showed a tendency to form hydroxyl adduct ions. Preliminary interpretations of the spectral data indicate these minor compounds are isomers or pentahydroxylated analogues of OA. Efforts to define the structural differences of these minor compounds are in progress.

The mass spectral analysis of OA has recently been expanded to include new derivative spectra and MS/MS. The spectra were compiled into a data set useful for analysts in identifying and quantifying OA in samples of biological origin. The EI and positive CI spectral patterns of the underivatized are compatible with the tetrahydroxylated cyclic heptaether structure. High resolution electron capture CI of the pentafluorobenzyl ester derivative confirmed the elemental composition of the carboxylate anion ($C_{44}H_{67}O_{13}$). Both the positive and negative FAB spectra confirmed

the molecular weight of 804 for the underivatized acid, and 818 for the methyl ester derivative. Significant qualitative and quantitative spectral changes were observed both in the simple and the MS/MS spectra upon addition of ammonium or alkali metal salts to the matrix, reflecting the acid's ionophoric nature.

In related work, a method has developed a method for the derivatization and HPLC-fluorometric determination of OA. The OA molecule possesses few structural features which absorb in the UV-VIS region and consequently detection by conventional spectrophotometric means are insufficient for detecting quantities of public health significance. A method for HPLC-fluorometric determination using 9-anthrylidiazomethane (ADAM) as a fluorescent derivatizing reagent has been proposed (Lee et al., 1987). However, the ADAM reagent is unstable in the solid and solubilized form and therefore must be prepared shortly before each use. The instability of ADAM renders it a cumbersome, time-consuming and potentially unreliable derivatizing reagent for routine usage. A new fluorescent labeling reagent, 1-pyrenyldiazomethane (PDAM) was reported to be stable as a solid for 5 years at -20°C for at least one week from preparation. In a recent study, the PDAM reagent was found to be a stable and useful tool, readily reactive with OA at 50°C without catalysis, providing a fluorescent ester, pyrenylmethylokadaate (PMO), easily detectable by HPLC-fluorometry. This analytical method was developed to determine the concentration of OA in laboratory cultures of P. concavum and P. lima. Okadaic acid in extracts of cells was esterified with PDAM in methanol-ethyl acetate (1:1). The fluorescent ester, PMO, was analyzed on a ODS column using acetonitrile-water (75:25) as eluant. The fluorescence

response was measured at an excitation wavelength of 340 nm and emission wavelength of 389 nm. The identity of the PMO peak was confirmed by mass spectrometry using desorption chemical ionization following trimethylsilylation. The standard curve generated with pure OA was linear over a range of 1-200 ng (r=0.9996). Analyses of culture extracts indicated that the cellular content of OA in P. concavum and P. lima increased with culture age, exhibiting maxima in late stationary phase of 8.14 pg/cell and 6.87 pg/cell, respectively. The performance and utility of the PDAM method for the determination of OA is currently being compared with a method recently developed by project 15827 utilizing 1-bromacetylpyrene (BAP) as fluorescent labeling reagent. Preliminary findings indicate the esterification of OA and analogues using BAP is more easily controlled because of the need for catalysis, and the product chromatograms contain fewer extraneous peaks that the ADAM or PDAM products.

Bioactives from dinoflagellate-associated bacteria

Bacterial isolations from dinoflagellate stock cultures and from recent field collections have yielded over 120 strains of bacteria for toxicological and related investigations. The bacteria collection has been characterized by traditional biochemical and susceptibility patterns which have resulted in a few typings to genus. Several of the bacterial strains have been determined to inhibit the growth of competing strains in culture. The bacterial collection is currently under evaluation for the presence of toxigenic strains and for influential affects upon dinoflagellate toxigenicity.